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Isoenzyme characterisation of Trichomonas vaginalis

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SUMMARY Clones of 32 strains of *Trichomonas vaginalis* isolated from patients attending a venereal diseases clinic were compared among themselves and with authentic *Pentatrichomonas hominis* on the basis of their isoenzyme patterns for eight enzymes by thin-layer starch-gel electrophoresis. The enzymes examined were: glucose phosphate isomerase (GPI); phosphoglucomutase (PGM); malic enzyme ($NADP^+$) (ME); hexokinase (HK); malate dehydrogenase (NAD^+) (MDH); glucose-6-phosphate dehydrogenase (G6PD); aldolase (ALD); and lactate dehydrogenase (LDH).

From the isoenzyme patterns of four enzymes (LDH, MDH, HK, and GPI) the strains of *T vaginalis* could be divided clearly into five groups. PGM showed differences in only one strain, while two other enzyme patterns (ME and ALD) were the same for all the strains of *T vaginalis* tested. All isolates were clearly distinguishable from *P hominis*. Although G6PD patterns were not sharp some differences were evident among *T vaginalis* strains.

Introduction

Teras[1] claimed that there was reason to believe that the human genitourinary trichomonad consisted of different types (possibly even different subspecies), on which depended the pathology and clinical course of trichomoniasis. A variety of immunological, serological, and pathological tests have been used to try to distinguish between different strains of *Trichomonas vaginalis*.[1-7] Although during the last few years some differences have been defined, very few data are available on the intraspecific variation in the biochemical activity of trichomonads and the relationship of these varieties to other biological properties. Isoenzyme techniques for the identification of isolates of parasitic protozoa, such as *Leishmania, Trypanosoma, Entamoeba, Plasmodium,* and others, have become essential in understanding the epidemiology of parasitic diseases, and there can be no doubt that biochemical analysis constitutes a fruitful area for future studies.

The aim of this work was to apply the technique of isoenzyme analysis (by thin-layer starch-gel electrophoresis) to the study of *T vaginalis*.

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Materials and methods

ORGANISMS

Specimens of vaginal fluid were collected, by a method similar to that of Robertson *et al*,[8] from female patients attending the venereal diseases clinic, Middlesex Hospital. Two mouse-virulent strains of *T vaginalis* were obtained from Italy by courtesy of Professor P Cappuccinelli; they were strain 1 (cryopreserved in London as LUMP 1192) and strain a20 (LUMP 1193). *Pentatrichomonas hominis* was obtained from the American type culture collection (ATCC 30000).

CULTIVATION

Human *T vaginalis* strains and the mouse-virulent strains were grown in the medium of Lumsden *et al*,[9] modified[10] by the replacement of liver digest by neutralised liver digest (Oxoid) and by altering the pH of the buffer component to 6·8. *P hominis* was grown in Stenton's[11] medium.

CLONING

Suspensions of cultured *T vaginalis* organisms were diluted and pour plates prepared by the method of Hollander.[12] The medium used was the TYM medium of Diamond,[13] modified by Hollander.[14] Dilutions were calculated to give 15-30 colonies per plate; plating efficiency was about 80%. Single

colonies were picked up and cultured further in liquid media.

PREPARATION OF EXTRACTS FOR ELECTROPHORESIS

The method used was basically that of Godfrey and Kilgour.[15] Organisms were harvested in the late exponential growth phase by centrifugation at 1000 × *g* for 30 minutes at 4°C and washed three times in phosphate-buffered saline. The washed pellets were lysed by mixing them with an equal volume of a hypotonic solution containing 1·0 mmol/l EDTA, 1·0 mmol/l dithiothreitol, and 1·0 mmol/l ε-aminocaproic acid and freezing. They were kept overnight at −20°C, thawed, and then centrifuged at 34 000 × *g* for 45 minutes at 4°C. The clear supernatant was removed and placed on ice; it was frozen as rapidly as possible in beads at −190°C until required or used immediately for electrophoresis.

ELECTROPHORESIS AND ENZYME STAINING

Electrophoresis was carried out as described by Wraxall and Culliford[16] and by Kilgour and Godfrey.[17] Modifications were made to improve the resolution of the system for use with trichomonad parasites. After electrophoresis the enzymes were visualised using formazan development in agar overlays by similar staining methods to Bagester and Barr[18]; controls were included for each enzyme by omitting the specific substrate. Substrates and developing reagents were obtained from Sigma. The conditions used in electrophoresis and the location of these enzymes are summarised in tables I and II. Soluble extracts from *T vaginalis* strains and other trichomonads were specifically stained for the following enzymes: (a) aldolase (ALD) E.C.2.4.1.13; (b) malic enzyme (oxaloacetate decarboxylating) (ME) E.C.1.1.40; (c) lactate dehydrogenase (LDH) E.C.1.1.1.27; (d) glucose phosphate isomerase (GPI) E.C.3.5.1.9; (e) phosphoglucomutase (PGM) E.C.2.7.1.1; (f) malate dehydrogenase (NAD^+ oxireductase) (MDH) E.C.1.1.1.37; (g) hexokinase (HK) E.C.2.7.1.1; and (h) glucose-6-phosphate dehydrogenase (G6PD) E.C.1.1.1.49.

Results

The results of electrophoresis of the enzymes examined are summarised in fig 1 and shown in fig 2. In most cases the enzyme patterns appeared within five minutes after specific staining and rapidly became diffuse on further incubation. The enzyme patterns were classified into two groups.

ENZYMES WITH SIMILAR ELECTROPHORETIC PATTERNS

Aldolase

All extracts of *T vaginalis* showed five separate, sharp, intense bands (figs 1*a* and 2*a*). The pattern of this enzyme was found to be constant, even when the electrophoretic conditions were slightly changed. The mouse-virulent strains showed the same patterns as normal *T vaginalis*.

Malic enzyme

The electrophoretic pattern of different strains of *T vaginalis* and the mouse-virulent strains can be seen in fig 2*b* and are summarised in fig 1*b*. Each extract showed three bands with a weak (fastest) one, which

TABLE I *Optimum conditions* for enzyme electrophoresis*

Enzyme	*Tank buffer (1 litre)*	*pH*	*Volts/cm across gel*	*Current (mA)*	*Gel buffer made from tank buffer*	*Running time (hours)*
GPI	0·122 mol/l Na_2HPO_4/0·078 mol/l NaH_2PO_4	7·0	16	15	1/13	2·5
PGM†	0·1 mol/l Tris/0·1 mol/l maleic/0·01 mol/l EDTA/ 0·01 mol/l $MgCl_2$	7·4	16	15	1/10	2·5
ME	0·122 mol/l Na_2HPO_4/0·078 mol/l NaH_2PO_4	7·0	16	15	1/10	3
HK	Stock: 0·45 mol/l Tris/0·01 mol/l EDTA/ 0·1 mol/l boric acid/0·0125 mol/l $MgCl_2$ Tank: dilute 1/7	8·9	20	20	1/10	3·5
MDH	0·05 mol/l phosphate/0·007 mol/l citric acid	7·0	20	20	1/13	3
G6PD	0·122 mol/l Na_2HPO_4/0·078 mol/l NaH_2PO_4	7·0	16	15	1/10	3
ALD	0·08 mol/l Na_2HPO_4/0·019 mol/l NaH_2PO_4	7·4	20	20	1/10	3·5
LDH†	0·1 mol/l Tris/0·1 mol/l maleic/0·01 mol/l EDTA/ 0·01 mol/l $MgCl_2$	7·8	20	20	1/5	3

*Temperature of the cooling plate was kept at 4°C throughout the run
†With reference to electrode buffers and PGM and LDH, the Tris, EDTA, and maleic acid components were first neutralised with NaOH in solution after which the $MgCl_2$ was added and the pH further adjusted with NaOH to the required value.
GPI = glucose phosphate isomerase; PGM = phosphoglucomutase; ME = malic enzyme; HK = hexokinase; MDH = malate dehydrogenase; G6PD = glucose-6-phosphate dehydrogenase; ALD = aldolase; LDH = lactate dehydrogenase; EDTA = ethylenediamine tetra acetic acid.

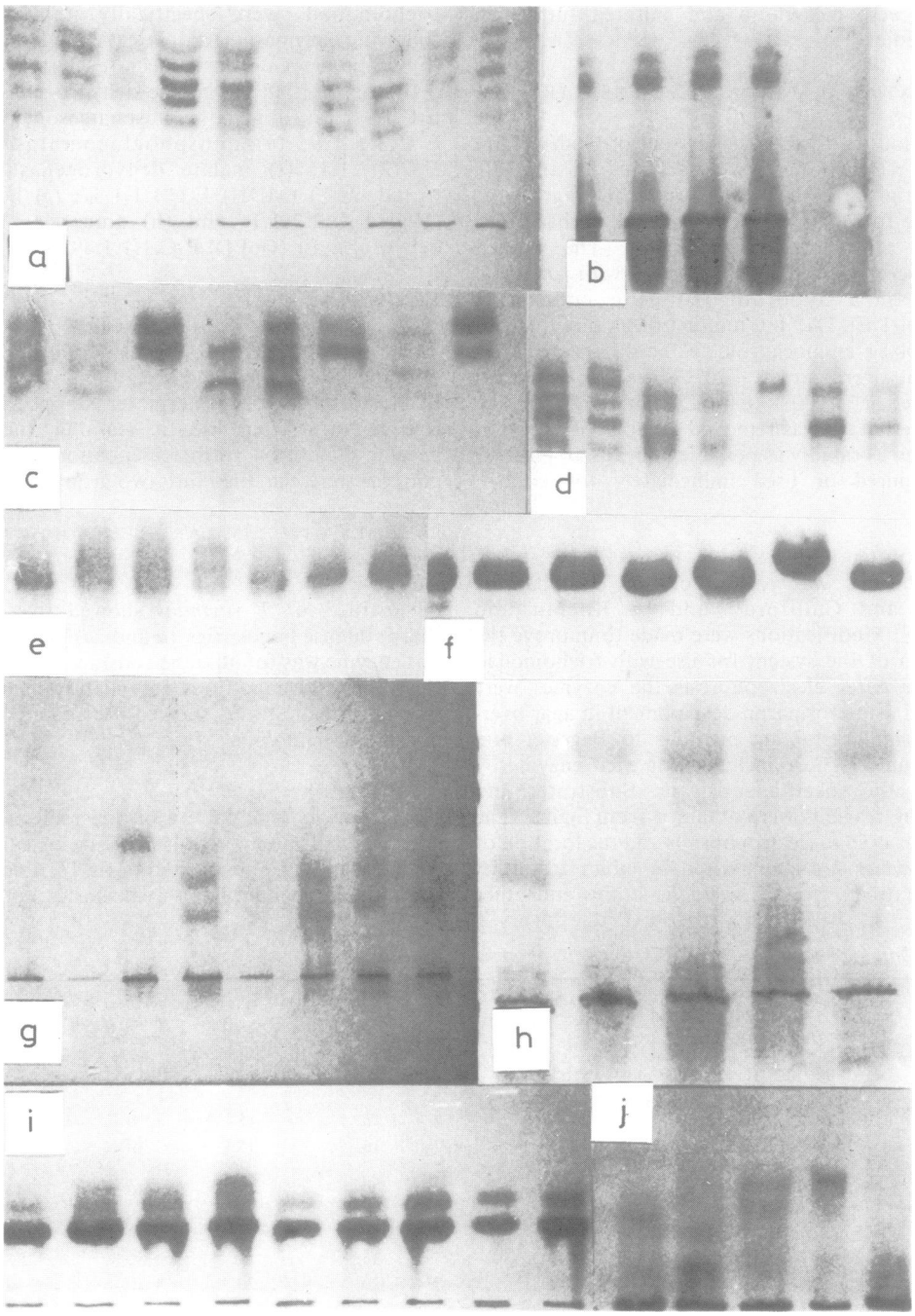

FIG 2 *Photographs of isoenzyme patterns (zymodemes) for the eight enzymes; (a) aldolase (ALD); (b) malic enzyme (ME) (oxaloacetate decarboxylating); (c) and (d) lactate dehydrogenase (LDH); (e) glucose phosphate isomerase (GPI); (f) phosphoglucomutase (PGM); (g) and (h) malate dehydrogenase (MDH); (i) hexokinase (HK); (j) glucose-6-phosphate dehydrogenase (G6PD).*

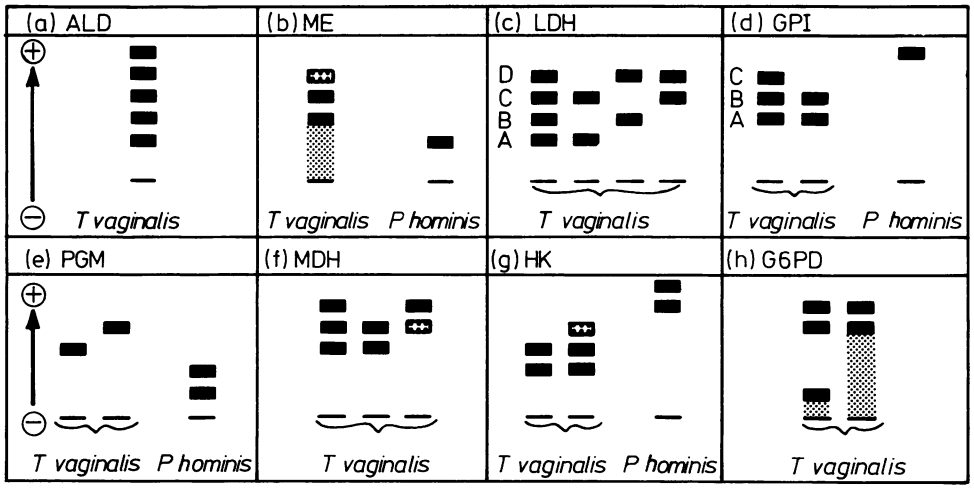

FIG 1 *Diagrams summarising enzyme electrophoretic patterns for: (a) aldolase (ALD) of various T vaginalis strains; (b) ME (malic enzyme "oxaloacetate decarboxylating") of various T vaginalis strains and P hominis; (c) lactate dehydrogenase (LDH) of various T vaginalis strains; (d) glucose phosphate isomerase (GPI) of various T vaginalis strains and P hominis; (e) phosphoglucomutase (PGM) of various T vaginalis strains and P hominis; (f) malate dehydrogenase (NAD^+ oxireductase) (MDH) of various T vaginalis strains; (g) hexokinase (HK) of various T vaginalis strains and P hominis; (h) glucose-6-phosphate dehydrogenase (G6PD) of various T vaginalis strains.*

developed later than the other two. Diffusion from the origin is characteristic of zymograms of this particular enzyme.

ENZYMES WITH DIFFERENT PATTERNS

Lactate dehydrogenase

Four different patterns among different extracts of *T vaginalis* were observed (figs 1*c*, 2*c*, and 2*d*); LDH I—pattern with four regular, sharp, intense bands (A, B, C and D) (samples 1 and 2 in fig 2*c* and 2*d*); LDH II—pattern with two sharp intense bands corresponding to bands A and C in pattern I (sample 4 in fig 2*c* and samples 3 and 4 in fig 2*d*); LDH III—pattern with two sharp intense bands corresponding to bands B and D in pattern I (fig 2*d*, samples 5, 6, and 7); LDH IV—pattern with two sharp intense bands corresponding to bands C and D in pattern I (fig 2*c*, samples 3, 6, 8).

Glucose phosphate isomerase (GPI)

T vaginalis strains usually gave patterns with three diffuse bands (A, B, and C; samples 2, 3, and 4 in fig 2*e*) or two bands (A and B; samples 5, 6, and 7 in fig 2*e*). One extract (mouse-virulent strain LUMP 1192), however, showed band A missing, while one other strain (LUMP 1242) showed an extra slow band.

Phosphoglucomutase

Almost all extracts of *T vaginalis* strains showed the same electrophoretic pattern with a single band, which was always in the same position and diffused within five minutes after development (figs 1*e* and 2*f*). Only one extract (mouse-virulent strain LUMP 1192) gave a band relatively faster than all the others (sample 6 in fig 2*f*).

Malate dehydrogenase

T vaginalis strains showed three different patterns (figs 1*f*, 2*g*, and 2*h*): MDH I—pattern with three bands (A, B, and C) (sample 4 in fig 2*g*); MDH II—pattern with two bands (as A and B in pattern I) (sample 6 in fig 2*g*); MDH III—pattern with two bands (as B and C in pattern I) (samples 2, 3, 4, and 5 in fig 2*h*). One of the two Italian mouse-virulent strains (LUMP 1192) showed a MDH III pattern while the other (LUMP 1193) showed a MDH I pattern.

Hexokinase (HK)

Two electrophoretic patterns were found (figs 1*g* and 2*i*): HK I—pattern with two intense bands (A and B) and a third weak band (C) (samples 2, 3, and 4 in fig 2*i*); HK II—pattern with two intense bands corresponding to bands A and B in HK I pattern but no band C (samples 5, 6, 7, 8, and 9 in fig 2*i*). The mouse-virulent strain, LUMP 1192, showed the HK I pattern while LUMP 1193 showed the HK II pattern.

TABLE II *Composition of enzyme developers*

	Developing conditions							
*Enzyme**	*Buffer*	*Substrate*	*Coenzyme*	*Other conditions*	*$MgCl_2$*	*MTT*	*PMS*	*Agar L28 (12 mg/ml)*
GPI	0·3 mol/l Tris/HCl, pH 8, 10 ml	10 mg/ml, F6P 1 ml	10 mg/ml NADP+ (4-Na-salt), 0·5 ml	100 U/ml G6PD solution, 500 µl	0·5 ml	2 mg	1 mg	10 ml
PGM	0·3 mol/l Tris/HCl, pH 7·4, 10 ml	25 mg G1P with 1% G1,6DP	10 mg/ml NADP+ (4-Na-salt), 0·4 ml	100 U/ml G6PD solution, 150 µl	0·5 ml	2 mg	1 mg	10 ml
HK	0·1 mol/l Tris/HCl, pH 7·4, 10 ml	450 mg D-glucose, 60 mg $ATP(Na)_2$	10 mg/ml NADP+ (4-Na-salt), 1·0 ml	100 U/ml G6PD solution, 10 µl	0·5 ml	4 mg	2 mg	10 ml
ME	0·3 mol/l Tris/HCl, pH 7·4, 8 ml	Malate solution† 1 ml	10 mg/ml NADP+ (4-Na-salt), 0·5 ml		2 ml	5 mg	2 mg	10 ml
MDH	0·3 mol/l Tris/HCl, pH 8, 6 ml	Malate solution† 4 ml	10 mg/ml NAD+ (di-Na-salt), 1 ml			2 mg	1 mg	10 ml
LDH	0·05 mol/l Tris/HCl, pH 8, 10 ml	Ca-lactate (Sigma) 35 mg	10 mg/ml NAD+ (di-Na-salt), 2·5 ml			5 mg	2 mg	10 ml
ALD	0·3 mol/l Tris/HCl, pH 8, 6 ml	F1,6DP 72 mg, glyceraldehyde-3-phosphate dehydrogenase 96 units	10 mg/ml NAD+ (di-Na-salt), 0·72 ml	0·3 mol/l Na-arsenate 1 ml; H_2O 2 ml		2 mg	1 mg	10 ml
G6PD	0·2 mol/l Tris/HCl, pH 8, 7 ml	G6P 5 mg	10 mg/ml NADP+ (4-Na-salt), 0·2 ml		3 ml	2 mg	1 mg	10 ml

*For full name, see table I
†10 mg/ml L-malic acid adjusted to pH 7·0 with 1·0 mol/l NaOH
F6P = fructose 6 phosphate; G1P = glucose 1 phosphate; G1,6DP = glucose 1,6 diphosphate; ATP $(Na)_2$ = disodium salt of adenosine 5'triphosphate; F1,6DP = fructose 1,6 diphosphate; G6P = glucose-6-phosphate; MTT = 3(4,5 dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide; PMS = phenazine methosulphate.

Glucose-6-phosphate dehydrogenase (G6PD)
The results with this enzyme gave some indication of two different types of electrophoretic patterns (figs 1*h* and 2*j*); however, as the bands tended to be very diffuse they were not included in the final classification. The electrophoretic patterns for the enzymes of *P hominis* tested are different from the other trichomonads and can be seen in fig 1*b, d, e,* and *g*). Using the enzyme patterns of LDH, MDH, HK, and GPI the *T vaginalis* may be divided into five groups (zymodemes) (fig 3 and table III).

TABLE III *Distribution of isolates* of T vaginalis among the five isoenzyme groups (zymodemes) defined in this study*

Group	*T vaginalis strain*
I	75/7479; 75/8040; 75/8095; 75/8555; 76/415; 76/2002; 76/7689; LUMP 1165; LUMP 1193; LUMP 1314
II	76/434; 76/991; LUMP 1031; LUMP 1042; LUMP 1043; LUMP 1315
III	75/8213; 76/714; LUMP 994; LUMP 1044; LUMP 1160; LUMP 1264
IV	75/2040; 75/8403; LUMP 1064; LUMP 1065
V	76/754; LUMP 996; LUMP 1046; LUMP 1232

*Of the 32 isolates examined, two (LUMP 1192 and 1242) were not assigned owing to anomalous patterns with GPI (both) and PGM (LUMP 1192).

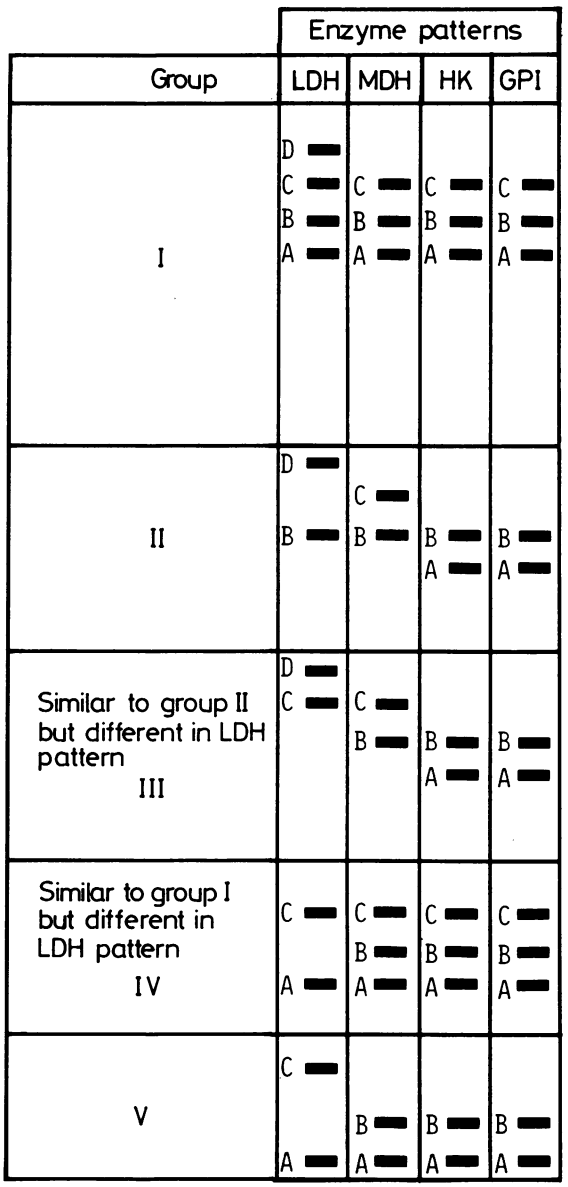

FIG 3 *Summary of the electrophoresis patterns found among T vaginalis isolates, which divide the organism into five groups (zymodemes).*

Discussion

In the present study isoenzyme analysis by thin-layer starch-gel electrophoresis was a promising method for the characterisation of trichomonads. It is a relatively simple technique and highly reproducible. It is important, however, that the isoenzyme patterns of several enzymes are examined when different strains of different trichomonads are being compared. In general, the isoenzyme patterns of all enzymes used in this study showed considerable differences between different trichomonad species when these were examined (unpublished data).

The fact that all strains of *T vaginalis* showed similar isoenzyme patterns of certain enzymes, for example, aldolase (ALD) and malic enzyme (ME), should not be regarded as a disadvantage; rather it tends to give confidence in the validity of the species.

At first the isoenzyme patterns of phosphoglucomutase (PGM) appeared to be similar in all *T vaginalis* strains tested; however, one of the two Italian strains (1192) showed a faster band. It is, therefore, possible that the enzyme will vary between strains. From the work done so far we cannot explain why some strains failed to show any bands at all in some enzymes. This could have been due to lack of activity, unsatisfactory conditions for the development, or because in *T vaginalis* and other trichomonads tested the enzyme is unusually labile. Another possibility is that the relatively crude extracts used might not be suitable for testing such enzymes. Our results with glucose-6-phosphate dehydrogenase would support the latter suggestion.

With this enzyme, while some strains of *T vaginalis* showed diffuse patterns, many others did not show any bands at all.

Among eight enzymes found to be satisfactory in the present study, lactate dehydrogenase (LDH) seemed to be very promising. The four different isoenzyme patterns (I, II, III, and IV) with this enzyme divided the *T vaginalis* strains into four groups. Interestingly, Teras and his collaborators[2] in Estonia divided his isolates also into four groups on the basis of antigenic type; it has not yet proved possible to see if the two classifications coincide.

With glucose phosphate isomerase (GPI), the electrophoretic patterns from most strains of *T vaginalis* fell into two groups. There is a possibility, however, of two further groups existing, because one of the (Italian) strains (1192) showed a missing band and another (LUMP 1242) showed an extra slower band which was not present in the other groups. Malate dehydrogenase (NAD^+ oxireductase) (MDH) is another enzyme which showed promising and reproducible results. *T vaginalis* strains could be divided into three different groups according to the electrophoretic pattern when using this enzyme. Possibly, more variability exists, because two forms (isoenzymes) of malate dehydrogenase (MDH) of one strain of *T vaginalis* were isolated by Brugerrolle *et al*[19] using sucrose density gradient and polyacrylamide gel electrophoresis. One form was located in the soluble fraction and the other was linked to the cytoplasmic "dense granules". Although these workers[19] have not compared different *T vaginalis* strains, their electrophoretic patterns of the soluble form of MDH were similar to one of the three patterns we found.

Using hexokinase (HK) enzyme we obtained two different electrophoretic patterns which divided *T vaginalis* strains into two groups. Comparison of these two groups with the three groups obtained by Teras *et al*[20] is difficult at this stage because they classified *T vaginalis* strains according to the total activity of the enzyme. Using this method of grouping they were able to correlate their results with virulence but not with antigenic differences among the 25 strains they used.

In the present study, using the enzyme patterns of LDH, MDH, HK, and GPI, *T vaginalis* was divided into five groups (zymodemes). While the importance of the present work cannot be wholly assessed until more is known about other properties which differ between zymodemes, at least there are now markers available to identify subpopulations of *T vaginalis* and distinguish them from other trichomonads. The enzymes tested in this study are unlikely to be the only ones suitable, and others can be looked for with some confidence. This method of identification offers the beginning of a biochemical classification to supplement the present morphological, immunological, and pathological classifications.

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